Journal of Cultural Heritage 15 (2014) 345–351

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Original article

A *Bacillus subtilis* cell fraction (BCF) inducing calcium carbonate precipitation: Biotechnological perspectives for monumental stone reinforcement

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ARTICLE INFO

Article history:
Received 5 June 2013
Accepted 3 October 2013
Available online 8 November 2013

Keywords:
Biomineralization
Calcareous stone
Weathering
Calcite
Bioconsolidation
Bacillus subtilis

ABSTRACT

Monumental stone decay is a consequence of the weathering action of physical, chemical and biological factors, which induce a progressive increase in porosity. To cope this degradation, bacterial calcium carbonate mineralization has been proposed as a tool for the conservation of monumental calcareous stones. The advantage of this kind of treatment is to obtain a mineral product similar to the stone substrate, mimicking the natural process responsible for stone formation. In this work, the possibility to induce $CaCO_3$ mineralization by a bacteria-mediated system in absence of viable cells was investigated and tested on stone. Our results showed that *Bacillus subtilis* dead cells as wells as its bacterial cell wall fraction (BCF) can act as calcite crystallization nuclei in solution. BCF consolidating capability was further tested in laboratory on slab stones, and in situ on the Angera Church, a valuable 6th century monumental site. New crystals formation was observed inside pores and significant decrease in water absorption (up to 16.7%) in BCF treated samples. A little cohesion increase was observed in the treated area of the Angera Church, showing the potential of this application, even though further improvements are needed.

1. Research aims

The main goal of our research was to identify alternative materials for consolidation and reinforcement of stone art works. In the last two decades, several biotreatments trials exploiting bacterial $CaCO_3$ precipitation have been done by using living cells and nutrient media to induce mineral precipitation into porous stones. We present a work in which we have isolated, characterized and tested a cellular fraction active in $CaCO_3$ precipitation, with the aim to reduce the organic matter applied on stone and bypass the negative side effects due to microbial growth.

2. Introduction

Monumental stone decay is a consequence of the weathering action of physical, chemical and biological factors, which induce a progressive dissolution of the mineral matrix and an increase in porosity, leading to weakening of the structure [1]. Inorganic or organic products have been used to slow down monument deterioration but their use presents several drawbacks [1,2]. Biomediated approaches to promote stone consolidation have been investigated and described. Bacterial calcium carbonate mineralization (BCCM) is a widespread process and represents a fundamental part of the calcium biogeochemical cycle, contributing to the formation of carbonate sediments, deposits and rocks [3–5]. BCCM has been proposed as a new tool in conservation of monumental calcareous stones firstly by a French group which developed the so-called Calcite Bioconcept technology [6,7]. The application of living cultures of selected biocalcifying bacterial strains can produce a superficial coating, called biocalcin [8]. The advantage of this treatment is to obtain a mineral product very similar to that of the stone substrate, mimicking the natural process responsible for calcareous stone formation. Several groups have been studying this system by testing different microorganisms, metabolic pathways and release

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http://dx.doi.org/10.1016/j.culher.2013.10.001

methods, prevalently on stone samples (slabs) in laboratory. The living calcifying bacteria used are either selected strains [1,8–12] or resident stone microbiota stimulated to precipitate calcite [13]. Other groups have been studying applications of living calcifying bacteria in remediation of concrete cracks or cementation in rock and construction materials. The activities and perspectives of research projects on stone bioremediation were recently reviewed [2,14,15]. These studies and applications all exploit precipitation occurring as a consequence of metabolic activities, one of the main mechanisms for BCCM [8,16]. Positive results in deposition of new calcite and in reduction of stone porosity have been obtained even if there is heterogeneity in the tests used for treatment evaluation [11]. However, application of viable bacteria and organic nutrients, supporting their growth, is not always appropriate for this kind of intervention. Uncontrolled growth of environmental microbes, such as airborne fungi, can be promoted by the supplied nutrients producing stained patches on treated stones [1], while a number of cells of the applied strains could be still viable in the biocalcite itself up to one year [12]. Further side effects, due to promotion of undesired microbial growth, include plugging of pores and formation of EPS, with possible aesthetical and mineral changes in the substrate [1,11].

For these reasons, the development of a stone treatment without viable cells seems a better biotechnological tool. In this view, attempts to induce calcite precipitation inside limestone have been made using organic molecules but the production costs are very high [17,18]. The problems with living bacteria and the past experience in using calcite bioinducing macromolecules (BIMs), led us to develop a bacteria-mediated system able to induce $CaCO_3$ mineralization in the absence of viable cells. This may exploit the positive traits of bacterial stone treatment bypassing the negative side effects due to microbial growth [19,20]. Our work has followed different experimental approaches in order to identify bacterial structure(s) and/or molecules inducing $CaCO_3$ precipitation. For all approaches, we have worked with *Bacillus subtilis*, a model laboratory bacterium that can produce calcite precipitates [1,21]. Previous experiments have shown that *B. subtilis* killed cells can still promote calcite formation [20]. Here we report results on the isolation of a *B. subtilis* cell fraction (BCF) active in inducing calcite precipitation. BCF has been tested on stones in laboratory conditions as well as on the Angera Church, a valuable 6th century monumental site. These results show this new biotechnology is worthy of being developed for stone reinforcement.

3. Materials and methods

3.1. Strains and growth conditions

B. subtilis strain 168 was used as test organism in this study. Maintaining medium: Nutrient Broth and Nutrient Agar (NB and NA, respectively, Oxoid). Precipitation growth medium: B4 medium (0.4% yeast-extract, 0.5% dextrose, 0.25% calcium acetate, 1.5% agar if solid [22]).

3.2. Preparation of killed cells

B. subtilis was grown at 37 °C in B4 liquid medium. Cells were collected during the exponential growth phase by centrifugation at 6,000 × g at 4 °C for 15 minutes, washed and resuspended in physiological solution (PS, 8.5 g l^{-1} NaCl), to obtain a concentration of 10^8 cells ml^{-1}. Suspension was then autoclaved at 121 °C for 30 minutes. Viable cells were counted before and after autoclaving. Cell suspension was stored at −20 °C.

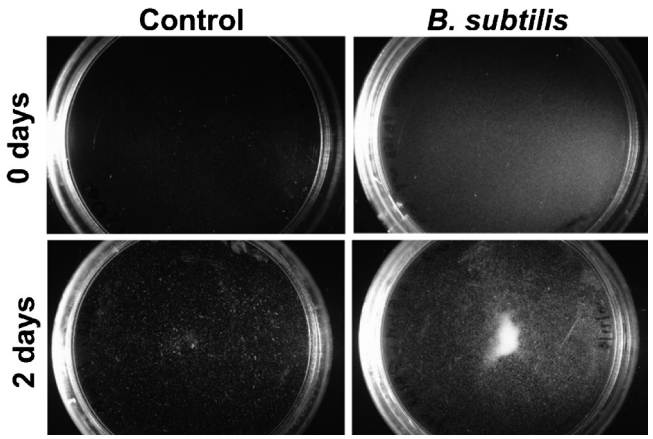

Fig. 1. Calcite precipitates obtained by *B. subtilis* 168 autoclaved cells. Bottom panel on the right shows a well visible crystal precipitation.

3.3. $CaCO_3$ precipitation test

For each sample, serial dilutions were prepared in PS, starting from the undiluted one corresponding to a concentration of 10^8 cells ml^{-1} for dead cells and to a concentration of 0.1 g ml^{-1} for bacterial cell fraction. 1 ml of each dilution was added to 9 ml of a 7.5 mmol l^{-1} $CaCl_2$ solution in a Petri dish (50 mm in diameter). Crystallization was induced by diffusion of ammonium carbonate vapors in a closed desiccator [23]. Incubation was at room temperature and samples were observed at the stereomicroscope (Olympus SZX9) for several days. $CaCl_2$ solution used as control, formed precipitates diffused in the Petri dish, with no progress after one-two days of incubation. Samples showing a precipitate comparable to that of the $CaCl_2$ solution were considered negative. Crystals produced by active samples were in larger amount prevalently concentrated in the middle of the Petri dish and increased with time (Fig. 1). No visible changes in crystal deposition in positive samples after the seventh day of incubation were observed.

3.4. Crystals analysis

Crystals obtained by $CaCO_3$ precipitation tests in $CaCl_2$ solution were collected by filtration through a Millipore filter (0.45 μm). Filters were dried at 60 °C over night. Precipitates were analyzed with Spectrophotometer FT-IR (Perkin-Elmer System 2000) and X-Ray diffraction equipment (Philips PW 1729).

3.5. Microscopy

The Light Microscopes (LM) used for bacteria and crystals analysis was the optical phase contrast Nikon Alphaphot YS; the scanning electron microscope (SEM) was JEOL JSM-840.

3.6. Cell stripping for cations

Bacterial cell wall surfaces were stripped of metal cations present in the growth medium [24]. After growth in liquid B4, cells were centrifuged at 6,000 × g for 15 min, rinsed two times in distilled, deionized (DDI) water, and soaked for 1 hour in 0.001 mol l^{-1} EDTA overnight, and finally rinsed five times in DDI water.

3.7. Subcellular fractionation

B. subtilis cells were grown in B4 liquid at 37 °C, collected during exponential growth phase and centrifuged 15 minutes at 6,000 × g at 4 °C. Pellet was washed with PS and resuspended as described

or stored at −20 °C. All the operations of grinding by alumina were carried out at 4 °C. Frozen pellet was grinded with an equal volume of alumina and grinded for 15 minutes. Then they were collected by adding 7.5 mmol l^{-1} $CaCl_2$ (about 10–15 ml per g of cells). After centrifugation at 2,500 × g, 4 °C for 20 minutes, to eliminate alumina and unbroken cells, the supernatant was further centrifuged at 10,000 × g, 4 °C for 15 minutes to separate the supernatant (cytosol) and pellet (membranes and cell walls). The supernatants and pellets were weighted and stored at −20 °C; this fraction was called *Bacillus* Cell Fraction (BCF). In order to proceed with the lyophilization, BCF pellet was resuspended in PS and frozen (Edwards Modulyo lyophilizer). The dry weight after lyophilization was usually 10% of wet weight. Freeze-dry BCF, or wet BCF pellet, was resuspended in 7.5 mmol l^{-1} $CaCl_2$ to 0.1 g ml^{-1} (original wet weight) before use.

3.8. BCF heat treatment

Resuspended BCF after lyophilization was divided in three aliquots: two were treated separately at 70 °C and at 100 °C for 10 minutes, the third aliquot was the untreated one. All samples were tested for $CaCO_3$ precipitation.

3.9. Stone samples

Stone specimens (size 10 × 10 × 4 cm) were obtained from a block of the Pietra d'Angera lithotype, a bioclastic stone constituted by dolomite with average porosity 18%.

3.10. Supersaturated solution

A supersaturated calcium bicarbonate ($Ca(HCO_3)_2$) solution (Super C), prepared by bubbling carbon dioxide (CO_2) through a calcium carbonate suspension was used for supplying calcium ions and CO_2 to the treated stones [18]. In order to maintain supersaturation in the pore and increase calcium ions, Super C solution was also supplemented with calcite nanoparticles (20 nm), at 2% w/v (SOCAL®, Solvay Ltd., UK).

3.11. Laboratory applications on stone samples

The lyophilized BCF was dissolved in 7.5 mmol l^{-1} $CaCl_2$ solution and then mixed with the Super C solution and sprayed on stone surface. Pietra d'Angera samples, in duplicate, were sprayed twice a day, with 2–3 hours interval, for 3 days on the same surface (100 cm^2) on a laboratory bench (average time of application: 10–15 minutes). During the treatments, stone samples were left drying overnight (two nights) in the same position. BCF was used at the concentrations described below and in volumes ranging from 25 to 35 ml for each application.

Spray application steps: the first day of treatment, BCF solution in Super C was sprayed (twice) at concentration of 5.5 g l^{-1} on two stone samples (BCF treated). Two stone samples were sprayed with Super C solution (called REF). The second and third day, BCF was sprayed (twice) in SuperC plus nanoparticles at final concentration 0.5 g l^{-1} on the two BCF treated samples, while only SuperC plus nanoparticles solution was sprayed on REF samples. The BCF concentration adopted for the test was derived from a similar application of organic matrix macromolecules [17].

3.12. Monument test site

A field test was performed on the Angera Cathedral (Chiesa di Santa Maria di Angera, Italy), which was built with the same Pietra di Angera stone used in laboratory tests [18]. The "Pieve of Angera" (Church of Angera, 6th century) is located in Angera (Lombardia, Italy). The field test was performed on the main façade (left side, South exposure), BCF (8.5 g l^{-1} in Super C solution) and REF (only Super C) were sprayed on the treated surface in selected areas of 0.29 m^2 and 0.28 m^2, respectively, for a total application of 1 l m^{-2} solution for each spray application. A non-treated surface of 0.04 m^2 was also used as control. BCF was used at the concentrations described below and in volumes ranging from 300 to 500 ml for each application.

Spray application steps: the first day of treatment BCF solution at concentration of 8.5 g l^{-1} in Super C and REF were sprayed on the selected areas. The second day BCF at final concentration 0.032 g l^{-1} in SuperC plus nanoparticles and REF plus nanoparticles were sprayed, while the third day only SuperC plus nanoparticles solution was sprayed on the selected areas. The amount of the sprayed BCF was increased for the *in situ* application due to the higher stone porosity when compared with the specimen used in laboratory experiments.

After 4 months, the field evaluation tests (surface color, cohesion profile and water absorption) were performed on treated and untreated areas. Furthermore, from each selected areas, a small core was collected for laboratory examination (penetration of the new calcite).

3.13. BCF treatment evaluation methods

Parameters considered for both laboratory and field stone treatments were: surface color, cohesion profiles, water absorption and production of new calcite.

The color of the selected area was determined by measuring the trichromatic co-ordinates with a Minolta Chroma Meter CR200 with light source type C. The same areas were measured before and after treatment by positioning the probe with a reference plastic sheet. The variation induced by treatments was determined calculating the ΔE [18].

The cohesion profiles were determined, before and after treatment, using the Drilling Resistance Measurement System (DRMS [18]) making 3 determinations for each area using the following operational conditions: revolution speed 600 rpm, penetration rate 10 mm min^{-1} and depth 10 mm. Diaber drill bit with 5 mm diameter.

Water absorption (Wa) was measured by the Contact Sponge method (CS) [18] using the following operative conditions: sponge loaded with 7 ml of water were applied for 60 seconds and pressed with an identical weight. Water was determined before and after treatment and expressed in mg cm^{-2} per minute as mean values of 3 measurements for each area.

Due to dolomitic composition of Pietra di Angera, the formation of new calcite crystals have been evaluated using the Alizarine Red S, a specific stain for calcite [18]. Thin sections were cut from the cores collected from each area of the monumental site, stained with a 0.025 M Alizarine Red S solution and observed under a LM (Nikon E600). The new calcite crystals are selectively red stained while the background of dolomite crystals remains unstained. The presence of the red spots due to the formation of new calcite inside the pores of the dolomitic Angera stone was evaluated for each thin section in 3 successive areas, from external to internal.

4. Results and discussion

4.1. Calcium carbonate production by dead B. subtilis cells

We previously demonstrated that *B. subtilis* dead cells can act as calcite crystallization nuclei (Fig. 1) [20,25]. To test if $CaCO_3$ precipitation was induced by cation adsorption on cell surfaces, *B. subtilis* cells grown in B4 medium and collected during exponential growth phase, were stripped for cations, autoclaved and tested for

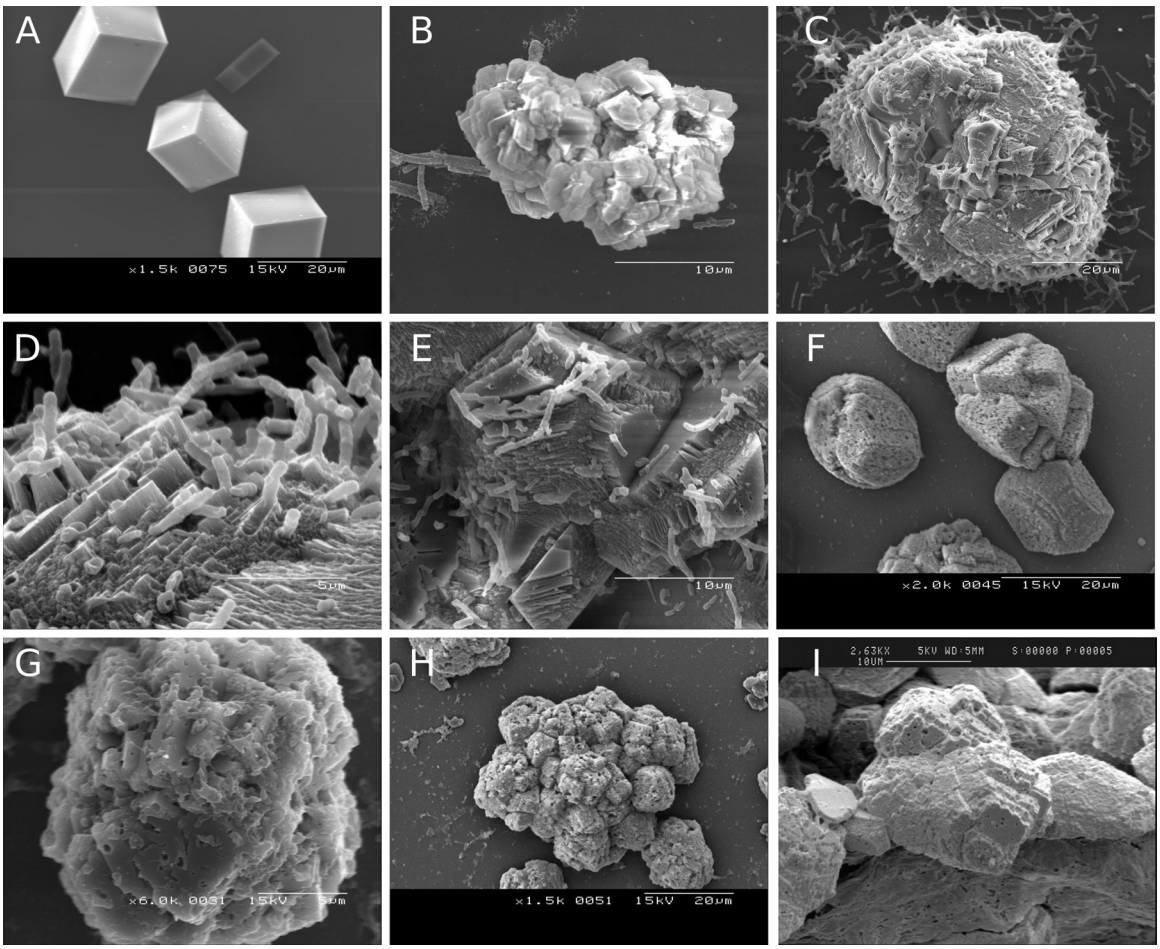

Fig. 2. SEM micrographs of calcite crystal produced by *B. subtilis* dead cells and BCF. A. Crystals formed in the control solution ($CaCl_2$). B–E. Crystal development by *B. subtilis* dead cells from day 1 to day 4. D, E. Crystal growth seems to be related to bacterial bodies. F–I. Calcite crystals induced by BCF.

precipitation activity. No differences were found in precipitation among stripped, stripped-autoclaved and autoclaved cells, showing that this capability was not due to cations adsorption on cells during growth.

4.2. Microscope observations of crystals formed by dead cells

Crystals produced by dead cells in solution were analyzed at the LM and at SEM after up to 5 days of incubation. Crystals formed in the control solution ($CaCl_2$ only) exhibited a single polarization color, indicating they are single crystals (data not shown), and a clear rombohedric shape both at LM and at SEM. All the crystals were single rombohedral crystals with a predominant size of about 10–20 μm (Fig. 2A) with some crystals up to 50 μm.

Crystals produced by *B. subtilis* autoclaved cells were always calcite of complex shapes based on intergrowth of rombohedrons. The different polarisation colors under the LM indicated that they were aggregates (data not shown). After one day of incubation, crystals were few and quite small with rombohedral morphology of single crystals (Fig. 2B). With time, they increased in size with an intergrowth of several single rombohedral crystals. The aggregates had a complex morphology and size up to 50 μm or more (Fig. 2C). Crystal growth seemed to be promoted by bacterial bodies, with cells aggregates arranged perpendicular as well as parallel to the growing crystal faces. Apparently, bacteria were arranged on the faces on which growth took place; these faces showed a superficial pattern with rombohedron edges, reflecting their crystalline orientation (001) (Fig. 2D). Bacteria appeared arranged with the main elongation axis perpendicular to the crystal faces, with cells included inside the crystallites (Fig. 2D). In other cases bacteria were arranged parallel to the crystal surfaces, suggesting a crystalline control on their attachment and orientation (Fig. 2E). These data are in agreement with the evidence that biomineralic carbonates exhibit highly unique morphologies not commonly seen on their inorganic counterparts [26].

4.3. Calcium carbonate production by bacterial cell fractions

B. subtilis 168 cells were fractioned to identify a cell fraction able to induce calcite formation. Cells were grinded by using alumina, as described in Materials and Methods. After centrifugation, two fractions were obtained: the cytosol (supernatant) was separated from cell wall, membranes and other debris (pellet). Both fractions were tested for precipitation in solution. The resuspended pellet always induced precipitation, while the supernatant never did. The activity was retained up to 1:8 dilution (undiluted sample 0.1 g wet weight ml^{-1}).

Crystals produced by *B. subtilis* active fraction (pellet) were analyzed by FT-IR spectrophotometry and X-ray diffraction to confirm calcite (no vaterite or aragonite were found).

The precipitation inducing ability of BCF is probably due to the presence of cell surface structures, in particular the cell wall component. Bacterial cell surface property to act as a site for cation adsorption and for heterogeneous nucleation is well known [27,28]. The *B. subtilis* cell wall, mainly composed of peptidoglycan and teichoic acids, is one of the most studied for interactions with metals

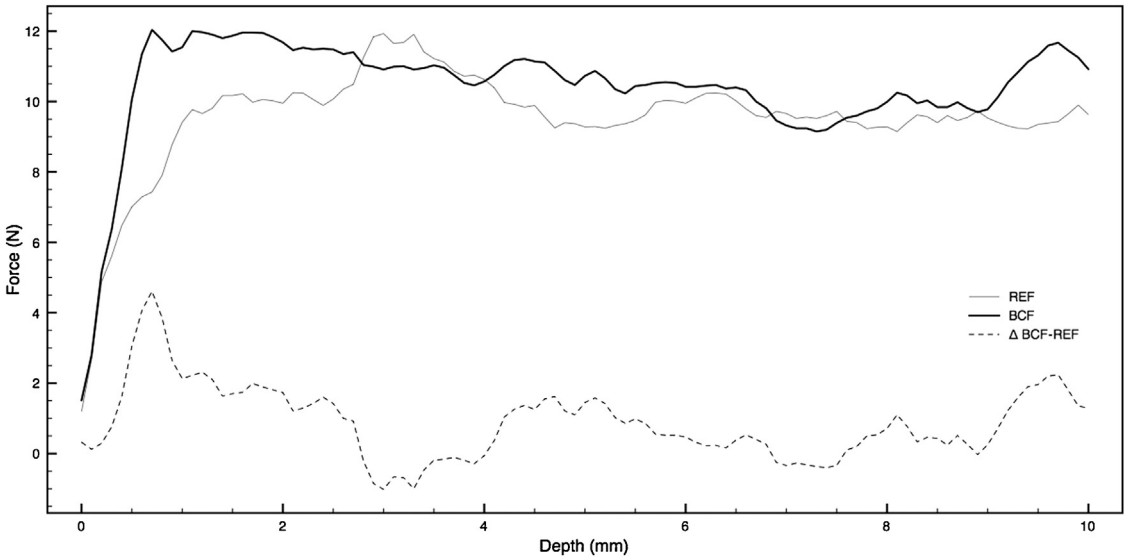

Fig. 3. Cohesion profiles from BCF and REF treated area of Angera Church. They were determined averaging the value of Drilling Resistance (DR) for three holes for each stone sample. Δ BCF-REF represents the difference between the two upper curves. Student's *t* test was inferred comparing the first 3 mm of penetration by BCF and REF (three replicas for each drill were taken). A statistically significant reinforcement was observed ($P < 0.036$) in the first 3 mm. The mean of the force required to drill the first three millimeters was 10.45 ± 0.37 N and 8.86 ± 0.38 N for BCF and REF, respectively.

[29]. Isolated walls of *B. subtilis* can uptake several metals and Ca^{2+} are among those strongly bound [30]. Moreover, functional groups on the cell walls of dead *B. subtilis* cells were considered able to complex dissolved Ca^{2+} in a calcite dissolution study [29].

There are few reports in literature, however, on bacterial dead cells as well as cell fractions inducing mineralization, except studies on outermost cell components as EPS [31]. Ben Omar et al. [32] reported that killed cells of *Myxococcus xanthus* can act as heterogeneous nuclei for struvite crystallization on solid medium. González-Muñoz et al. [33] reported struvite and calcite crystallization induced by cellular membranes of *M. xanthus* only on solid mineral medium. They discussed the lack of calcite crystallization in liquid medium to be in agreement with the occurrence of calcite crystal nucleation on inducing proteins adsorbed onto a rigid substrate, as found by Addadi and Weiner [23]. In our study, bacterial dead cells as well as BCF can act as heterogeneous crystallization nuclei in liquid medium even after autoclaving or cell disruption treatment, probably retaining the rigid scaffold of the cell wall. This biomineralization process can be referred to biologically-influenced mineralization, according to Dupraz et al. [5].

Finally, to test long-lasting activity, lyophilized BCF preparations were stored at 4 °C and 18 °C and tested after different storing times. Precipitation activity was maintained after a storage period (both at 4 °C and 18 °C) up to 6 months (the longest tested period). In addition, preparation activity was also maintained after two heating treatments, at 70 °C and 100 °C, indicating that BCF active components were heat resistant. Tests were repeated twice on two different BCF samples.

4.4. Microscope analysis of crystals formed by BCF

Crystals produced by BCF in solution were analyzed by using LM and SEM. The crystals showed different polarization colors under the LM (data not shown), which indicated they were aggregates instead of single crystals. In the aggregates, crystals with rombohedral and excalenoedral morphologies could be identified (Fig. 2F). The morphology of crystal aggregates was globular (Fig. 2G, H). They were constituted of different amounts of crystals radially distributed. Firstly, many nucleation sites appeared and, after their stabilization, the aggregates were generated (Fig. 2G, H). The globular aggregates exhibited a porous surface (Fig. 2F–I) and a size up to 50 μm, with single crystals ranging between 5 and 20 μm (Fig. 2H). Crystals produced by BCF were also aggregates of globular shape, with a tendency to link among them. Moreover, the presence of BCF induced larger nucleation, respect to the control solution, by lowering the super saturation grade needed for a heterogeneous nucleation. They resemble calcite crystals produced by *B. subtilis* living cells on solid as well as in liquid B4 precipitation medium [1,34]. At least for *B. subtilis*, calcite crystals obtained by both biologically-influenced and biologically-induced mineralization show a similar globular morphology, typical of biogenic calcite minerals [35].

4.5. Test on stone specimens (in vitro) and on the façade of S. Maria of Angera's Cathedral (in situ)

A BCF solution at a maximum concentration of 5.5 g l^{-1} was applied by spray on Pietra d'Angera samples and 8.5 g l^{-1} on the main façade of S. Maria of Angera's Cathedral, built with the same Pietra di Angera stone used in laboratory tests (same quarry of origin), respectively. Treatments were evaluated by using three different methodologies: color of the surface (to assess aesthetical changes), water absorption (to estimate crystal precipitation) and drilling resistance (to measure the hardness of the treated samples). Color changes due to the treatments were negligible for both the *in vitro* and *in situ* experiments, being the ΔE lower than 3 (detection limit for human eye is ΔE = 3). Sixteen decimal seven percent and 6.8% decrement in water absorption was observed for the *in vitro* and *in situ* experiments, respectively (Table 1). The water absorption in the controls remained substantially unchanged (Table 1). With reference to the drilling resistance, the mean value was determined by the DRMS (3 holes for each area, treated and REF). No significant differences were reported for the *in vitro* samples. On the opposite, the cohesion profiles indicated a significant increase in hardness in the first 3 mm (Fig. 3) in the Angera Church area treated by BCF, up to 4 N if compared with the REF treated area. This little cohesion increase is most probably due other than to the higher BCF concentration used for monument application rather than for laboratory treatment (8.5 g l^{-1} versus 5.5 g l^{-1} in vitro application) even to the fact that the stone material present on the

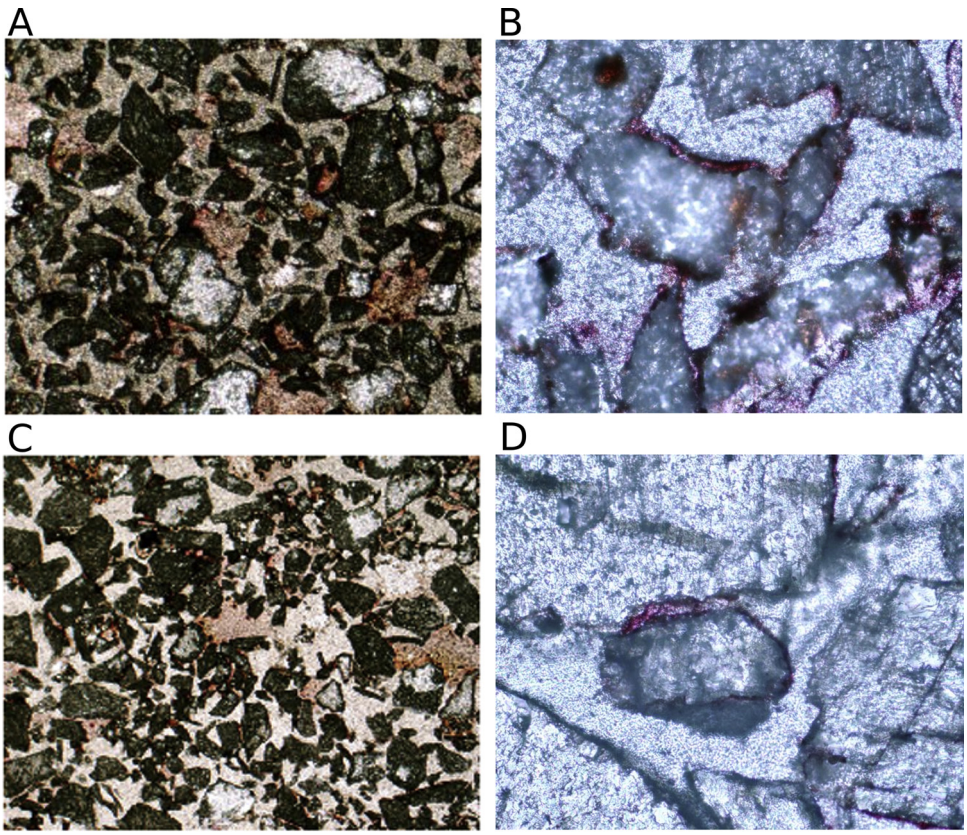

Fig. 4. Representative thin sections made from cores taken from stone slabs of the Angera Cathedral stained with Alizarine red and observed under LM. A, B. Angera's stone treated with BCF (40× and 400×, respectively). C, D. Angera's stone treated with REF (40× and 400×, respectively). Red areas represent the new calcite formation (visible online).

Table 1
Water absorption by contact sponge. Numbers express the water absorption, Wa = mg cm^{-2} min^{-1}, and their standard deviations before and after the treatment in parenthesis. Δ represents the difference in term of percentage between before and after the treatment. Results are reported as mean values of 3 measurements for each stone sample.

Treatment	Water adsorption		Δ %
	Before treatment (mg cm^{-2} min^{-1})	After treatment (mg cm^{-2} min^{-1})	
REF (Lab)	41.3 (1.9)	40.1 (2.2)	2.9
BCF (Lab)	43.4 (2.1)	36.2 (1.9)	16.7
REF (Ang)	40.1 (2.2)	39.4 (2.7)	1.6
BCF (Ang)	39.4 (2.5)	36.7 (2.1)	6.8

Angera church is more degraded than the specimen used for the laboratory experiments and thus the DRMS tests can evidence the little increase due to the new calcite precipitated.

For the same reason, when *in situ* stone samples were stained with Alizarine Red solution, new calcite formation inside the pores was observed by using a LM. New calcite formation induced by BCF was displayed very abundant if compared with the REF sample (Fig. 4).

In agreement with Webster and May [11], BCCM technology is still in its infancy and, therefore, not readily available, even if results seem promising. Evaluation of stone treatments with biocalcifying bacteria or molecules have been studied using a wide heterogeneity of different techniques [8–12]. In order to compare the effectiveness of biotreatments of stones, we would like to stress the importance in developing standard methods for evaluating changes in porosity and strengthen in relation to biological calcification.

5. Conclusions

In our knowledge, this is the first report on the isolation of a bacterial cell fraction able to induce calcite precipitation in solution and on its use in bioreinforcement tests on specimen as well as on monumental stones. Our main results show that a bacterial fraction containing the cell wall is active in mineral precipitation without the contribution of cellular metabolism. We encountered encouraging results in terms of reduced water absorption and cohesion profile on treated surfaces which fit with the new calcite deposition inside stone pores. Nevertheless, further experimentation is needed to evaluate the effectiveness of the BCF treatment in stone consolidation and work out the treatment conditions suitable for monumental stone applications. Finally, this kind of treatment is innovative respect to other experimented "biotreatments" because it avoids the application of living cells and nutrient media needed for microbial growth on stone. This technology is in particular worthy of being developed for treatment of calcareous statues and objects of high aesthetic and artistic value where conservation is required with the minimum change in their chemistry [18].

Acknowledgments

We want to thank Mrs C. Indorato for technical assistance. This work was supported by EC grant EVK4-CT2000-00037 "BIOREINFORCE".

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